AEM
Journals.ASM.org

Identification of Critical Genes for Growth in Olive Brine by Transposon Mutagenesis of *Lactobacillus pentosus* C11

G. Perpetuini,[a,b] H. Scornec,[b] R. Tofalo,[a] P. Serror,[d,e] M. Schirone,[a] G. Suzzi,[a] A. Corsetti,[a] J. F. Cavin,[b] H. Licandro-Seraut[b,c]

Dipartimento Scienze degli Alimenti, Università di Teramo, Teramo, Italy[a]; UMR A 02102 PAM, AgroSup Dijon, and Université de Bourgogne, Dijon, France[b]; Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, Paris, France[c]; INRA, UMR1319 Micalis, Jouy-en-Josas, France[d]; AgroParisTech, UMR Micalis, Jouy-en-Josas, France[e]

Olive brine represents a stressful environment due to the high NaCl concentration, presence of phenolic compounds known as antimicrobials, and low availability of nutrients. Thus, only a few strains of lactic acid bacteria (LAB) are adapted to grow in and ferment table olives. To identify the mechanisms by which these few strains are able to grow in olive brine, *Lactobacillus pentosus* C11, a particularly resistant strain isolated from naturally fermented table olives, was mutagenized by random transposition using the P_{junc}-TpaseIS*1223* system (H. Licandro-Seraut, S. Brinster, M. van de Guchte, H. Scornec, E. Maguin, P. Sansonetti, J. F. Cavin, and P. Serror, Appl. Environ. Microbiol. 78:5417–5423, 2012). A library of 6,000 mutants was generated and screened for adaptation and subsequent growth in a medium, named BSM (brine screening medium), which presents the stressful conditions encountered in olive brine. Five transposition mutants impaired in growth on BSM were identified. Transposition occurred in two open reading frames and in three transcription terminators affecting stability of transcripts. Thus, several essential genes for adaptation and growth of *L. pentosus* C11 in olive brine were identified.

Table olives represent one of the most important fermented vegetables, as they are widespread both in the Mediterranean area and in Australia, the United States, and South America; they are highly appreciated for their sensory characteristics and nutritive value, and their use is extensive in all markets. Even if table olives represent an important economic source for the producing countries, the fermentation process is still empirical and craft based (1). Table olives can be elaborated in several ways, but the main industrial elaborations are the green Spanish style and the naturally black olives, or Greek style. These treatments aim to remove the natural bitterness of olives through the hydrolysis of oleuropein. In the Spanish style bitterness is removed by adding lye (NaOH solution), while in the Greek style olives are directly brined and the debittering is slow and only partial (1, 2). These processes are unpredictable and can lead to low-quality products. Many studies have focused on standardizing the quality of products through the use of starter cultures composed of lactic acid bacteria (LAB) and yeasts (3–8). Among yeasts, the species proposed as starters are *Candida diddensiae* (3), *Saccharomyces cerevisiae* (9), and *Debaryomyces hansenii* (10). Regarding LAB, *Lactobacillus plantarum* (11–13) and *Lactobacillus pentosus* (14, 15), which are very closely related species, are the main bacteria used as starters. They possess general stress resistance genes that have been identified in *L. plantarum* for other food fermentations (14). During the olive fermentation process, an ecological succession of different strains of these two species takes place. These strains have different biochemical and physiological characteristics and are adapted to the particular conditions of olive brines (15–17). In recent years, the interest of researchers was focused on the study of the antimicrobial effects of phenolic compounds present in table olives (18–20). Particular attention was paid to *L. plantarum*, while only few analyses were performed with *L. pentosus* (20). Some bacterial responses to phenolic compounds were deeply characterized, particularly the phenolic acid stress response (PASR) in *Pediococcus pentosaceus* (21), *L. plantarum* (22–24), and *Bacillus subtilis* (25, 26), which allows the bacteria to face ferulic, *p*-coumaric, and caffeic acid stresses.

The species of LAB encountered most frequently during table olive fermentation are *L. plantarum* and *L. pentosus* (17). Effectively, previous studies demonstrated that, at the end of fermentation of Itrana Bianca table olives (naturally fermented green table olives), the bacterial population was clearly dominated by *L. pentosus* and *L. plantarum* species (11–13). The mechanisms involved in the ability of these strains to grow in such a hostile environment are not known. So, the aim of this study was the identification of genes involved in the capability of *L. pentosus* C11 to adapt and to grow in olive brine and thus to perform olive fermentation. Therefore, by using random transposon mutagenesis, one of the most powerful approaches widely used for genetic characterization of bacterial phenotypes (25, 27, 28), a library of 6,000 random transposon mutants was generated using the P_{junc}-TpaseIS*1223* system (29) and screened for the ability to grow in olive brine, using BSM (brine screening medium).

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Multiplex PCR on the *recA* gene was performed to check the assignment of strain C11 to the *L. pentosus* species, as previously described (30). Wild-type (WT) *L. pentosus* C11 and its corresponding mutants were routinely grown either in liquid or in agar MRS medium (20 g/liter; Difco) at 37°C without shaking, whereas for several growth ability tests, liquid or agar (20 g/liter) YG medium composed of 10 g/liter yeast extract and 10 g/liter glucose was used as the basal medium. *Escherichia coli* cells were grown aerobically at 37°C in Luria-Bertani (LB) medium. Relevant antibiotic concentrations were as follows: 150 μg/ml erythromycin (Em) and 50 μg/ml ampicillin (Am) for *Esche-*

Received 10 April 2013 Accepted 14 May 2013
Published ahead of print 17 May 2013
Address correspondence to J. F. Cavin, cavinjf@u-bourgogne.fr, or H. Licandro-Seraut, Helene.Seraut@u-bourgogne.fr.

doi:10.1128/AEM.01159-13

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
L. pentosus C11	Isolated from table olives	University of Teramo culture collection
E. coli TG1	*supE hsd*Δ*5 thi* Δ (*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	54
Plasmids		
pVI129	Ap^r Cm^r, pVI1056 containing P_{hlbA}-IS*1223*ΔIR	29
pVI110	Em^r, pBR322*ori*, P_{junc}	29

richia coli, and 10 μg/ml chloramphenicol (Cm) and 5 μg/ml Em for recombinant strains of *L. pentosus* C11 (Table 1). The olive-extracted phenolic compounds mix used for phenotypic analysis of selected mutants was obtained through high-performance liquid chromatography purification of olive oil vegetation waters (31) and contained 131 mg/g 3,4-dihydroxyphenylethanol (3,4 DHPEA), 20 mg/g *p*-hydroxyphenylethanol (*p*-HPEA), 64 mg/g verbascoside, 165 mg/g 3,4-DHPEA-EDA (dialdehydic form of elenolic acid linked with 3,4-dihydroxyphenylethanol), and 381 mg/g other phenolic compounds.

Development of BSM. A solid medium called brine screening medium was developed in this study to isolate mutants affected in their ability to grow in olive brine. In particular, the brine was taken from the end of fermentation of Itrana Bianca table olives (naturally fermented green table olives), when antimicrobial phenolic compounds reach the highest concentration. The main characteristics of this brine were previously described (2, 32). In particular, the brine (70 g/liter NaCl, pH 4.0) was mainly characterized by the presence of 3,4-DHPEA, *p*-HPEA, and verbascoside (32), and also glucose and lactic acid (2). In order to obtain a 100% concentration of brine in the agar medium, brine was 2-fold concentrated with a rotary Buchi vacuum apparatus at 52°C and then supplemented with 20 g/liter glucose and 20 g/liter yeast extract as nutrients. The pH was adjusted to 4.0 with HCl, corresponding to the general olive brine pH. Then, the supplemented brine was pasteurized at 65°C for 45 min, a treatment sufficient to destroy any vegetative microorganisms (determined by agar plating [data not shown]), and mixed with an equal volume of sterile melted agar at 40 g/liter in water just before pouring the plates. Mutants were spotted on BSM plates (diameter, 150 mm) by using a 96-solid-pin replicator and incubated for 72 h at 37°C. YG medium at pH 6.0 was used as the positive growth control. Absence of growth on BSM plates was confirmed in a replicate experiment.

DNA techniques. All DNA manipulations were performed according to standard procedures (33). Plasmids were isolated by using a Nucleospin plasmid miniprep kit (Euromedex). Ligation and restriction analysis were carried out according to the manufacturer's instructions. PCR was performed using 0.1 unit of Platinium *Taq* DNA polymerase high fidelity (Invitrogen), according to the manufacturer's recommendations, in an automatic thermocycler (Bio-Rad).

Transposon mutagenesis. *L. pentosus* C11 and *E. coli* were transformed by electroporation as described by Aukrust et al. (34) and Dower et al. (35), respectively, using a GenePulser and a pulse controller apparatus (Bio-Rad). Plasmids were prepared from *E. coli* by using a Nucleospin plasmid miniprep kit (Euromedex). Mutagenesis was performed using the P_{junc}-TpaseIS*1223* system as previously described (29) with the following modifications. Electrocompetent cells of *L. pentosus* C11 were first electroporated with pVI129, which provided the transposase of IS*1223*, plated onto MRS agar with Cm at 10 μg/ml, and incubated at 37°C for 48 h. Electrocompetent cells of a pVI129 transformant were then transformed with pVI110. The cells were plated on MRS agar plates supplemented with 5 μg/ml Em and incubated at 42°C for 48 h to select integrants.

TABLE 2 Primers

Use and name[a]	Sequence (5′→3′)
pVI110 target sequencing	
IRR6	TCACCGTCATCACCGAAACG
IRL6	GCCGCACTAGTGATTAAAATAC
qRT-PCR	
obaA F	GATTGGGCTCGTCCTAGTCA
obaA R	TATTGTTGGAAGCCGTCGAT
obaB F	CTTGACCGTTGTCACCCAAT
obaB R	AGAAACAGTGCGGGTTCAAA
gpi F	ATCGGGATTGGTGGTTCATA
gpi R	TGTGGGAATTTACGGTCTTCA
obaC F	CGTTTAACAACGCTGAGCAA
obaC R	AACCCTTCACCACAACAAGC
obaE F	ATAGCGACAGCACCTGCAC
obaE R	CGATGATTTGGTCACGGAAC
obaF F	TCAGGCACCATAAGCATCG
obaF R	TGCAACCGAATTAACAGGA
enoA1 F	GCAAGGTAGTCCGTGTTCGT
enoA1 R	ACTGGGAAGACTGGCAAATG

[a] F, forward; R, reverse.

Sequence analysis and mapping of transposon insertion site. Genomic DNA was digested simultaneously with ClaI and BstBI (Fermentas), and ligation was performed using T4 DNA ligase HC (Fermentas) according to the manufacturer's instructions. The resulting ligation products were directly transformed into the *E. coli* TG1 strain, in which circularized fragments that contain the transposon replicate as plasmids. Plasmids were isolated from selected transformants and subjected to sequencing reactions (GATC Biotech) using the primers IRR6 and IRL6, which target the transposon sequence extremities (Table 2). Identification of transposon target sequences was performed with the BLAST software from the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/).

RNA extraction and qRT-PCR analysis. Total RNA from 10 ml of culture was extracted after bead beating disruption using the Tri reagent method (Sigma) as previously described (24). Quantitative reverse transcriptase PCR (qRT-PCR) and calculations of relative transcript levels (RTLs) were carried out as previously described elsewhere (24), using the primers listed in Table 3. The genes *tpiA* and *rpoD* were used as internal calibrators for all qRT-PCR analyses.

RESULTS

Transposon mutagenesis of *L. pentosus* C11. *L. pentosus* C11, isolated from olive brine (unpublished data), was mutagenized

TABLE 3 Analysis of transposon integration in 10 randomly selected *L. pentosus* C11 mutants based on sequencing of the transposon target and BLAST analysis with the *L. pentosus* IG1 genome sequence

Mutant no.	Locus of pVI110 insertion
1	IGR *lpent_00526/lpent_00527*
2	ORF *lpent _00833*
3	ORF *lpent_00835*
4	IGR *lpent _01101/lpent _01102*
5	IGR *lpent _01190/lpent _01192*
6	IGR *lpent _01719/lpent _01720*
7	IGR *lpent _01772/lpent _01773*
8	ORF *lpent _02891*
9	IGR *lpent_02977/lpent _02978*
10	IGR *lpent_03116/lpent_03117*

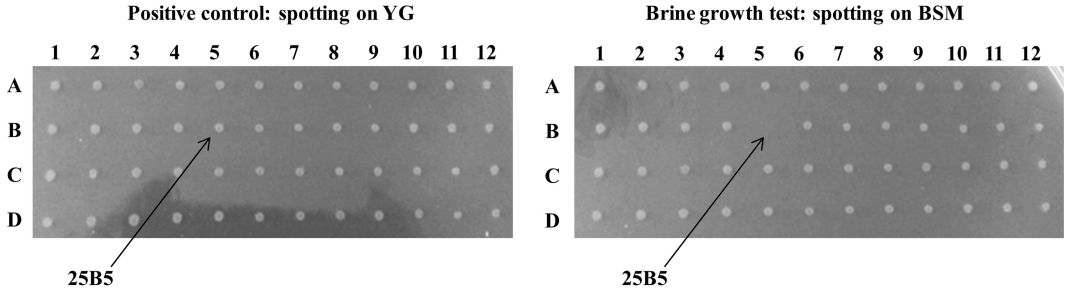


FIG 1 Photographs of plates obtained during screening of 48 *L. pentosus* C11 mutants for their sensitivity to olive brine after replication from fresh titration microplate individual cultures on either YG or BSM and incubation at 37°C for 72 h.

using the P_{junc}-TpaseIS*1223* system, which was specially designed for LAB random transposon mutagenesis and successfully used for *Lactobacillus casei* mutagenesis (29). This system is composed of the thermosensitive pVI129 plasmid, which is used for transient expression of the IS*1223* transposase and the nonreplicative plasmid pVI110, which contains dedicated inverted repeat sequences that require the transposase for pVI110 random integration into the genome. The segregational stability of pVI129 in *L. pentosus* at 42°C was estimated to be 80% per generation by using the calculation method of Heap et al. (36). Thus, the mutagenesis procedure applied for *L. pentosus* was the same as that described for *L. casei*, but the final incubation was at 42°C. With approximately 4,000 mutants obtained from 10^9 viable cells (carrying pVI129) transformed with 1 μg of pVI110, the transposition efficiency was similar to that of *L. casei* (29). Less than 10 Em^r colonies were obtained for WT *L. pentosus* electroporated with pVI110, confirming the necessity for pVI129 to promote pVI110 integration. Eighteen pVI110 integration mutants were randomly selected and analyzed by Southern blotting with a pVI110-specific probe as previously performed (29). Southern blot analysis indicated that pVI110 was integrated randomly at a single locus of genomic DNA of *L. pentosus* C11 (data not shown). This was confirmed by sequencing of the genomic DNA target of pVI110 in 10 randomly selected mutants (Table 3). The transposon was integrated into different loci, strongly supporting the randomness of pVI110 integration in *L. pentosus*. Thirty percent of the mutants were disrupted in open reading frames (ORFs) (Table 3). Moreover, the target sequence of pVI110 was reanalyzed for 5 of the 10 randomly selected mutants after 50 generations and gave the same results, demonstrating the stability of pVI110 integration. Altogether, these results confirmed the efficiency and the randomness of the transposon mutagenesis in *L. pentosus* when using the P_{junc}-TpaseIS*1223* system. Thus, a collection of 6,000 colonies, in which about one-third of the mutants in an ORF are expected, were randomly picked and prepared in 96-well plates for phenotypic screening.

Screening for mutants unable to grow on olive BSM. In order to select mutants from the mutagenized library of *L. pentosus* C11 affected in their capacity to grow in brine, a solid agar medium named brine screening medium was developed by supplementing YG-agar medium with olive brine (see Materials and Methods). Mutants of the library were spotted on BSM plates, with a positive growth control on YG plates (Fig. 1). After 48 h of incubation at 37°C, of the 6,000 mutants screened, 5 failed to grow on BSM but grew on YG plates. This inability was also confirmed in BSM broth (BSM without agar): there was no increase of the optical density at 600 nm (OD_{600}) monitored during the 48 h following the inoculation at an OD_{600} of 0.1.

Phenotypic analysis of the selected brine-sensitive mutants. Phenolic compounds and osmotic stress triggered by salt concentration, which can reach 70 g/liter, are the main stresses encountered in olive brine. To establish the main factors responsible for mutant growth inhibition in brine and to better understand the niche adaptation phenomenon, the growth of the 5 selected mutants was compared to that of WT *L. pentosus* under the main stress conditions encountered in olive brine. Bacterial growth was tested in YG broth (pH 4) containing an increasing concentration of NaCl and supplemented or not with either oleuropein (0.2 g/liter) or an olive-extracted phenolic compounds mix (0.4 g/liter). Only a few differences between the growth of WT *L. pentosus* and mutants were detected in YG broth at pH 4 after 24 h, in the absence of stress conditions (Fig. 2) or with 10 g/liter NaCl (data not shown), with a similar final OD_{600} (about 1.3) for WT *L. pentosus* and mutants. These results demonstrated that the identified mutants were not sensitive to acidic conditions nor to 10-g/liter NaCl supplementation. The growth of all mutants was partially inhibited with 20 g/liter NaCl (Fig. 2), and none of the 5 mutants was able to grow in the presence of 30 g/liter NaCl or higher concentrations, contrary to findings with WT *L. pentosus* (data not shown).

Contrary to WT *L. pentosus*, the growth of the 5 brine-sensitive mutants was affected in the presence of the olive-extracted phenolic compound mix (Fig. 2). This result agrees with that obtained for *L. pentosus* ATCC 8041, which was able to grow in the presence of different phenolic compounds, such as hydroxytyrosol and its glucosides, oleoside, tyrosol, secoxyloganin, secologanoside, and oleuropein (20). Supplementation with 20 g/liter NaCl increased the inhibition triggered by phenolic compounds for all mutants (final OD_{600} of about 0.3), whereas it had no consequence on WT *L. pentosus* growth (Fig. 2). These observations confirmed that the inability of the mutants to grow in brine results from the combined stress induced by phenolic compounds and NaCl, as reported for other LAB strains not well adapted to this inhospitable environment (37). For example, oleuropein, one of the most abundant phenolic compounds in olive pulp, exerts an inhibitory effect on bacterial growth by a mechanism that remains unknown (20). It is suspected to induce leakage of glutamate and inorganic phosphate from bacterial cells, cause degradation of the cell wall itself (19, 38), and also cause a decrease in the ATP content of cells (39). The growth of the five brine-sensitive mutants was inhibited at the same level, with a final OD_{600} of 0.6 in YG containing 0.2 g/liter oleuropein, with or without 20 g/liter NaCl (Fig. 2). Notice-

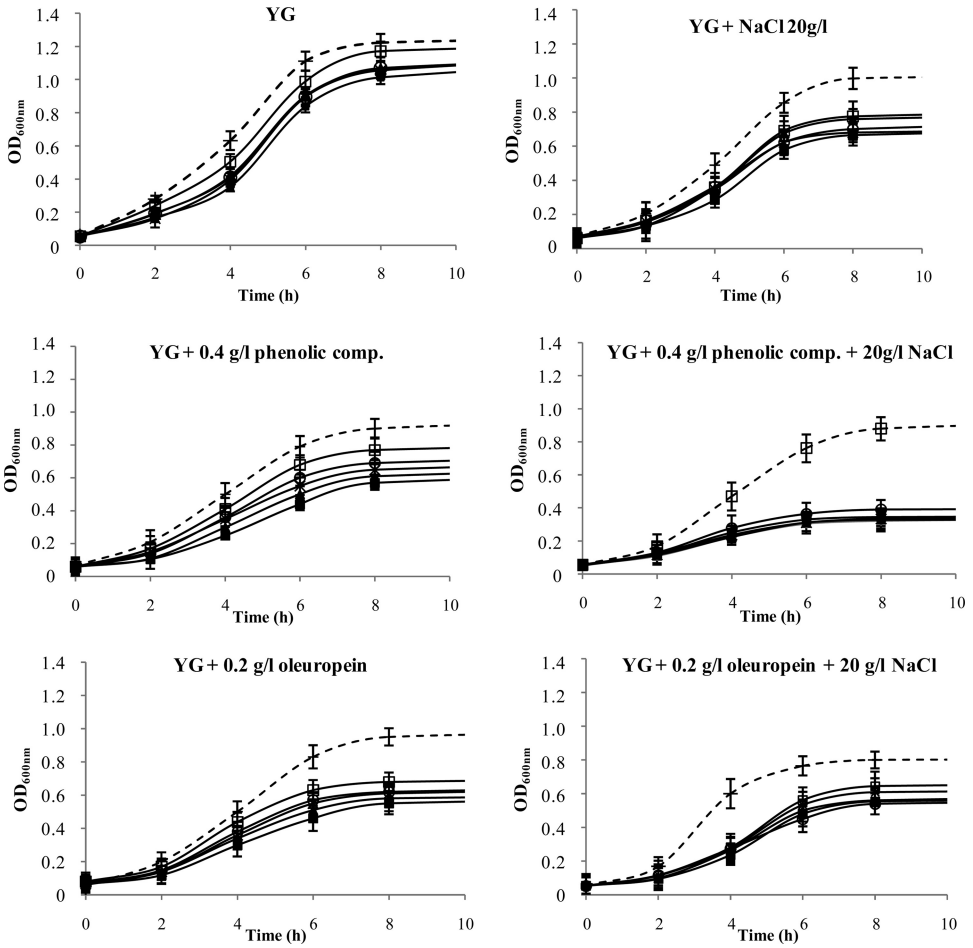


FIG 2 Growth of WT *L. pentosus* C11 (dotted line connected plus signs) and brine-sensitive mutants 25B5 (■), 20B10 (▲), 31B11 (×), 51D12 (○), and 42G1 (□) under different stress conditions with phenolic compounds, oleuropein, and NaCl.

ably, the inhibition of the mutant was particularly strong during the first hours of growth, in comparison with WT *L. pentosus* (Fig. 2).

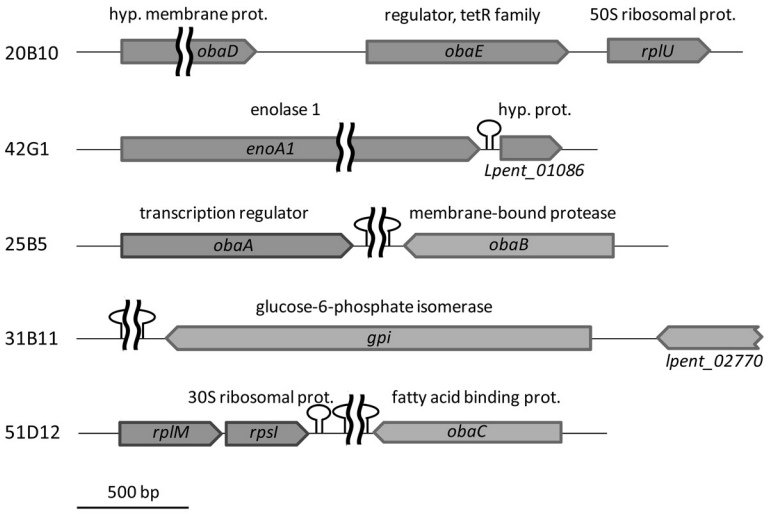
From phenotype to olive brine adaptation genes. Transposon chromosomal targets were determined by sequencing and comparison of the insertion site sequence with *L. pentosus* IG1 genomic sequence using BLAST database searches (NCBI). The disrupted genes were named *obaA* to *obaE*, for olive brine adaptation, when no putative function was already attributed.

In mutant 20B10, the transposon was inserted in *obaD*, an ortholog of *lpent_00392*, which encodes a small hypothetical integral membrane protein of unknown function. The *obaD* gene seems to be specific to *L. pentosus/L. plantarum* species, since neither ortholog nor even homologs of the ObaD-encoded protein were found in other bacterial species. This gene is located 379 bp upstream of *obaE*, an ortholog of *lpent*_00391, which encodes a transcriptional regulator of the Tet^r family. Despite the quite large intergenic region (IGR) between *obaD* and *obaE*, no putative transcriptional terminator was found in this IGR, and RT-PCR with two primers designed for *obaD* and *obaE*, respectively, on WT *L. pentosus* also indicated that *obaD* was cotranscribed with *obaE* (data not shown). The consequence of transposon integration on *obaE* was evaluated by qRT-PCR performed on mutant cultures grown in MRS until the early stationary phase of growth, using WT *L. pentosus* under the same culture condition as the reference (Fig. 3). In the 20B10 mutant, *obaE* transcripts were under the detection level (lower than 1/1,000 of that measured for the WT), indicating that *obaE*, in addition to *obaD*, is silenced in this mutant.

The mutant 42G1 is disrupted for *enoA1*, an ortholog of *lpent_01085*, which is predicted to encode an enolase (EnoA1), an essential enzyme for glycolysis (Fig. 3). *L. pentosus* C11 is likely to carry another enolase gene, as is the case for the sequenced *L. plantarum* strains, which would explain the fact that this disruption is not lethal. The RTL of the gene downstream of *enoA1*, the *lpent_01086* ortholog, was not modified by the transposon integration in *enoA* (RTL of 1), and a putative transcription terminator was found downstream of *enoA1* (Fig. 3).

In mutants 31B11, 51D12, and 25B5, the transposon was integrated into the predicted transcription terminators (TT) (Fig. 3). In mutant 31B11, the transposon was integrated into the TT of *gpi*, an ortholog of *lpent*_02771, which encodes glucose-6-phosphate isomerase, whereas in mutant 51D12 it is integrated into the TT of *obaC*, an ortholog of *lpent*_00851, which encodes a putative fatty acid binding protein. The transposon of mutant 25B5 was integrated into a TT that could serve for the two convergent genes

A



B

Mutant	Gene	RTL mutant vs WT
20B10	*obaD*	ND (disrupted)
	obaE	<1/1000
42G1	*enoA1*	ND (disrupted)
	lpent_01086 ortholog	1
25B5	*oaA*	<1/1000
	obaB	<1/1000
31B11	*gpi*	1/6
	lpent_02770 ortholog	1
51D12	*rpsI*	1
	obaC	<1/1000

C

Mutant	Transposon insertion site sequences
20B10	TTTTAGTCAGTTTTGTTAGTTTCTTTATCTTT/TGATTGTATAATGCCAATGTGCAAGATAAGAA
42G1	AGGGTGAAGGCGAAAATGGTCAATCATTTACT/GTGCCGGTTACAAGCCTGGTAAGGATATTGCC
25B5	GAAAGCGGGATATTGTTGCCACATAGGTAAGA/AATTATTCAGGGAAGCTTTTTTAACATACTTA
31B11	AAGCCCCCAAAACAAGCAGCCTATCAGCCAGC/TGATAGTTGTTTGTTTTAGGAGCTTCCATCTT
51D12	GTTCAGCATTTGAAGCTGAACTGAAAGTTATG/TCATATAGCCAGAAAATGTTGATAAAACAACG

FIG 3 (A) Schematic representation of transposon integrations in the 5 olive brine-sensitive mutants. Transposon integration is represented by a vertical, curved double line. (B) RTLs of genes of the locus affected by the transposon, using WT *L. pentosus* C11 as the reference. ND, not determined (gene disrupted). (C) Transposon insertion site sequences.

obaA, an ortholog of *lpent*_01150, which encodes a putative redox-sensitive transcription regulator, and *obaB*, an ortholog of *lpent*_01149, which encodes a putative membrane-bound protease.

Olive brine sensitivity of IGR integrants could be due to polar effects of the transposon on the flanking genes. To check this hypothesis, qRT-PCR analysis of genes flanking the transposon was performed for each mutant, using WT *L. pentosus* as the reference (Fig. 3). The RTL of *gpi* in mutant 31B11 was 6-fold lower than in the WT, while transposon insertion did not change the RTL of the upstream gene, the *lpent_02770* ortholog. The lower *gpi* RTL could be explained by an alteration of the transcript stability, as bacterial single-stranded RNA is rapidly degraded from the 3′ end (40, 55). The consequence should be a decrease of the glucose-6-phosphate isomerase (GPi) concentration in the cell, which could alter carbohydrate-related metabolic functions. As Gpi plays a central role in carbohydrate metabolism, a stronger reduction of the *gpi* RTL, or its inactivation by transposon insertion, would have been lethal. Interestingly, these results illustrate an unexpected aspect of IGR mutants, in which in some cases transposon integration into a TT reduces the transcript level of an essential gene without affecting growth and viability under nonstressful conditions. The transcript level of *obaC* was under the detection level in mutant 51D12 (<1/1,000 of that in WT), while the RTL of the divergently transcribed gene, *rpsI*, did not change (Fig. 3). This is consistent with the presence in the IGR for these two genes of two independent hairpin structures predicted to be transcription terminators. The drastic reduction of the *obaC* RTL could be due to instability of *obaC* mRNA presenting an altered TT. Furthermore, a hairpin structure downstream of *obaC* displays high similarity level with the RF01676/P31 noncoding RNA family (http://rfam.sanger.ac.uk/). Interestingly, in mutant 25B5, transposon insertion reduced drastically the RTL of the two flanking genes, *obaA* and *obaB*. No alignment with noncoding RNA of a known family was found, but their presence cannot be excluded. From a functional point of view, 51D12 can be considered an *obaC* knockout mutant and 25B5 as a double *obaA-obaB* knockout mutant.

Despite the fact that we did not obtain two or more mutants in each of the five genes identified in this screening, indicating that testing of 6,000 mutants is not sufficient to reach saturation, our drastic screening conditions allowed us to identify some of the most essential genes for growth in olive brine.

Transcriptional analysis of the genes interrupted, or silenced by transposon integration, was carried out on WT *L. pentosus* after 16 h of growth in BSM broth, or in YG broth for the reference condition (Fig. 4). All genes identified in olive brine-sensitive mutants displayed a significantly higher RTL in BSM broth than in YG broth, with particularly high increases for *enoA1* (60-fold) and *obaE* (35-fold) (Fig. 4). This analysis reinforces their role in the resistance to the olive brine stress and in olive fermentation adaptability and demonstrates that this adaptation results in an upregulation of *oba* gene transcription in WT *L. pentosus*.

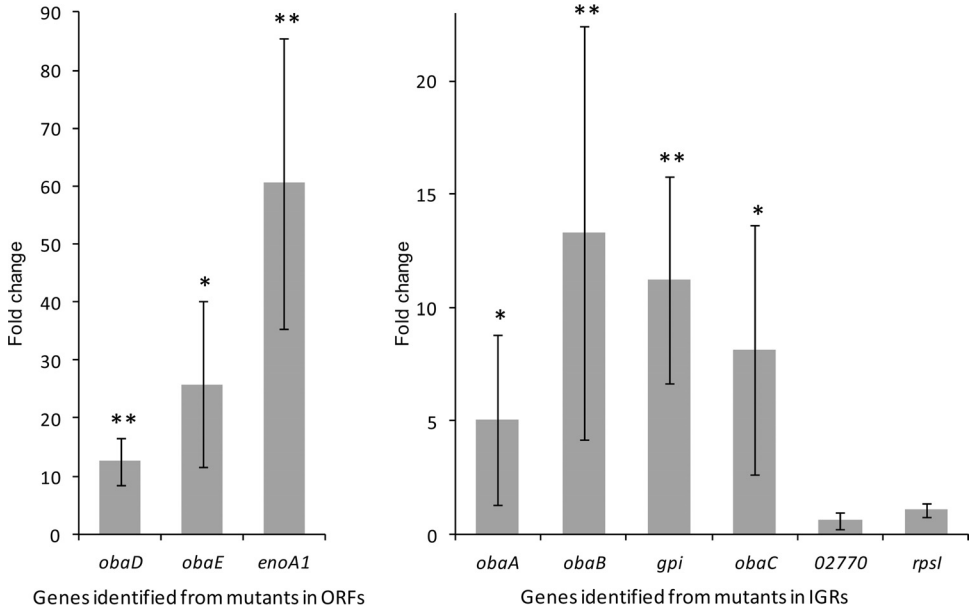


FIG 4 Relative transcript levels of *L. pentosus* C11 genes after 16 h of growth in BSM broth. Transcript levels of each gene are expressed as the relative fold change, with YG medium as the reference condition (fold change = 1). Four biological repeats were performed, and bars indicate standard deviations. Statistical analysis was performed using the unpaired Student *t* test: *, $P < 0.05$; **, $P < 0.005$.

DISCUSSION

Olive brine represents a stressful environment due to the high NaCl concentration, presence of phenolic compounds considered antimicrobials, and low availability of nutrients. It was observed that the inhibitory effect of olive phenolic compounds on LAB growth was higher when they were associated with NaCl, showing a combined effect in the inhibition (37).

Oleuropein and hydroxytyrosol (the product of oleuropein hydrolysis) are the most abundant phenolic compounds found in olives, and they show antimicrobial effects (20). The mechanisms of bacterial growth inhibition have not been elucidated, and often controversial results have been obtained, depending on the different antimicrobial assays used (19). However, several studies agreed that oleuropein and its hydrolysis products could impact cell wall structure. These compounds could induce leakage of glutamate and inorganic phosphate from bacterial cells, as well as the degradation of the cell wall itself (19, 38) and changes in the typical bacillary structure of Gram-positive bacteria (38). Moreover, oleuropein has been reported to cause a decrease in the ATP content of the cells without affecting the rate of glycolysis (39).

The response to osmotic stress implies involvement of several genes whose expression, coordinated by osmosensing regulation, leads to an adjustment of cytoplasmic properties, cell turgor, hydration, and thus protein activity (42, 43). The sequence analysis of *L. pentosus* IG1, a strain isolated from Spanish-style green olive fermentations, revealed that this strain presents 16 putative two-component regulatory systems, which may reflect an extensive ability to adapt to changing environmental conditions (56).

The stress response in LAB and particularly in *Lactobacillus* has been reviewed recently (44). The genes involved in the generalized stress response in Gram-positive bacteria and LAB appear to be highly conserved (45). Thus, the ability of only some strains of *L. plantarum* and *L. pentosus* to face olive brine stresses is probably related to the presence of a genotypic diversity in the "lifestyle adaptation island," which is a chromosomal region suggested to be involved in niche adaptation (45).

The strategy used in this work allowed us to select 5 mutants of *L. pentosus* C11 unable to grow in olive brine because of the simultaneous presence of inhibitory concentrations of NaCl and the phenolic compounds that characterize this medium.

Thanks to genetic and transcriptomic analyses of mutants, *enoA1*, *gpi*, and *obaC* were clearly identified as essential genes for growth in olive brine. Concerning mutants 20B10 and 25B5, our results did not discriminate between *obaD* versus *obaE* or *obaA* versus *obaB*, respectively, since these mutants can be considered double knockouts and since these four genes are upregulated in the presence of olive brine. Further studies are required to establish which one of *obaA* or *obaB* is needed for adaptation to olive brine.

Gene expression analysis demonstrated that all *oba* genes are upregulated in the presence of olive brine, reinforcing the evidence of their involvement in brine stress resistance and growth in this medium. These genes can be classified into three groups: the first group includes *gpi* and *enoA1*, both of which encode enzymes involved in energetic metabolism; the second group includes *obaB*, *obaC*, and *obaD*, which encode putative membrane proteins; the third group includes *obaA* and *obaE*, which encode proteins with a putative regulatory function.

Under stress conditions, carbon metabolism, which is involved in energy production and saving, can be disturbed, as reported for *L. plantarum* challenged with tannic acids (46) and for *Lactobacillus rhamnosus* in the presence of acid stress (47). The enolase gene is generally one of the upregulated metabolic genes in response to stress; for instance, its expression is induced with decreasing temperatures of growth in *Lactobacillus helveticus* (48) and in a low-pH environment for *Lactobacillus reuteri* (49). So, enolase gene disruption, as well as a glucose-6-P-isomerase (*gpi*) RTL decrease, could affect the efficiency of metabolic pathways involved

in stress adaptation. Amazingly, enolase 1 has also been described as a surface protein able to bind fibronectin in *L. plantarum* (50), indicating that other roles for enolase 1 in sensing the environment or in interacting with olive brine molecules are possible.

The *obaD/obaE* operon encodes two putative proteins of unknown function. ObaD, a putative membrane protein without an identified conserved domain, is exclusively found in *L. plantarum* and *L. pentosus* species, while ObaE is a putative transcription regulator of the Tet^r family. Thus, ObaD/ObaE could constitute an element of a system for sensing and responding to environmental changes like osmotic pressure variations and appearance of phenolic compounds. This is consistent with previous work that indicated that phenolic compounds are responsible for alterations of the cell wall and modifications of fatty acid membrane composition in *L. plantarum* (51).

The *L. plantarum* ortholog of *obaB*, which encodes a membrane-bound protease CAAX family protein, is upregulated in the presence of ethanol (52). It has been proposed that this protein could be involved in the lipid ordering and bilayer stability and it could influence permeability, fluidity, and the functioning of membrane-embedded enzymes (52). Regarding *obaC*, which encodes a putative DegV family protein, its cellular function remains unknown, but it could be involved in lipid transport or in fatty acid metabolism (53).

In this study, five mutants unable to grow in olive brine were isolated, and their analysis led to the identification of at least 5 genes essential for the growth of *L. pentosus* under olive fermentation conditions, which are characterized by a particularly high concentration of salt and phenolic compounds. The identified genes encode proteins involved in carbohydrate metabolism, membrane structure and function, or regulation of gene expression. As *obaD* encodes a putative membrane protein strictly specific to *L. pentosus/plantarum* species, it is one of the key elements that deserves further characterization in order to understand the particularly efficient adaptation mechanisms in these two closely related species to environments that present these specific stresses, which are found in many fermented food processes and also natural ecosystems.

ACKNOWLEDGMENTS

This work was part of the project 580 ''Tracciabilitaà, certificazione e tutela della qualità dell'olio di oliva e delle olive da tavola—Azione 4d'' supported by grant 582 from UNAPROL (867/2008 Misura 4). G. Perpetuini was the beneficiary of a grant financed by the European Social Fund (FSE). This work was also supported by the Scientific Council of AgroSup Dijon and Conseil Régional de Bourgogne.

REFERENCES

1. **Aponte M, Ventorino V, Blaiotta G, Volpe G, Farina V, Avellone G, Lanza CM, Moschetti G.** 2010. Study of green Sicilian table olive fermentations through microbiological, chemical and sensory analyses. Food Microbiol. **27:**162–170.
2. **Tofalo R, Schirone M, Perpetuini G, Angelozzi G, Suzzi G, Corsetti A.** 2012. Microbiological and chemical profiles of naturally fermented table olives and brines from different Italian cultivars. Antonie Van Leeuwenhoek **102:**121–131.
3. **Hurtado A, Reguant C, Bordons A, Rozes N.** 2010. Evaluation of a single and combined inoculation of a *Lactobacillus pentosus* starter for processing cv. *Arbequina* natural green olives. Food Microbiol. **27:**731–740.
4. **De Bellis P, Valerio F, Sisto A, Lonigro SL, Lavermicocca P.** 2010. Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. Int. J. Food Microbiol. **140:**6–13.
5. **Panagou EZ, Schillinger U, Franz CM, Nychas GJ.** 2008. Microbiological and biochemical profile of cv. *Conservolea* naturally black olives during controlled fermentation with selected strains of lactic acid bacteria. Food Microbiol. **25:**348–358.
6. **Perricone M, Bevilacqua A, Corbo MR, Sinigaglia M.** 2010. Use of *Lactobacillus plantarum* and glucose to control the fermentation of "Bella di Cerignola" table olives, a traditional variety of Apulian region (southern Italy). J. Food Sci. **75:**M430–M436.
7. **Corsetti A, Perpetuini G, Schirone M, Tofalo R, Suzzi G.** 2012. Application of starter cultures to table olive fermentation: an overview on the experimental studies. Front. Microbiol. **3:**248.
8. **Botta C, Cocolin L.** 2012. Microbial dynamics and biodiversity in table olive fermentation: culture-dependent and -independent approaches. Front. Microbiol. **3:**245.
9. **Segovia Bravo KA, Arroyo Lopez FN, Garcia Garcia P, Duran Quintana MC, Garrido Fernandez A.** 2007. Treatment of green table olive solutions with ozone. Effect on their polyphenol content and on *Lactobacillus pentosus* and *Saccharomyces cerevisiae* growth. Int. J. Food Microbiol. **114:**60–68.
10. **Tsapatsaris S, Kotzekidou P.** 2004. Application of central composite design and response surface methodology to the fermentation of olive juice by *Lactobacillus plantarum* and *Debaryomyces hansenii*. Int. J. Food Microbiol. **95:**157–168.
11. **Leal-Sanchez MV, Ruiz-Barba JL, Sanchez AH, Rejano L, Jimenez-Diaz R, Garrido A.** 2003. Fermentation profile and optimization of green olive fermentation using *Lactobacillus plantarum* LPCO10 as a starter culture. Food Microbiol. **20:**421–430.
12. **Lamzira Z, Asehraou A, Brito D, Oliveira M, Faid M, Peres C.** 2005. Reducing the bloater spoilage during lactic fermentation of Moroccan green olives. Food Technol. Biotechnol. **43:**373–377.
13. **Marsilio V, Seghetti L, Iannucci E, Russi F, Lanza B, Felicioni M.** 2005. Use of a lactic acid bacteria starter culture during green olive (*Olea europaea* L cv Ascolana tenera) processing. J. Sci. Food Agric. **85:**1084–1090.
14. **Bron PA, Wels M, Bongers RS, van Bokhorst-van de Veen H, Wiersma A, Overmars L, Marco ML, Kleerebezem M.** 2012. Transcriptomes reveal genetic signatures underlying physiological variations imposed by different fermentation conditions in *Lactobacillus plantarum*. PLoS One **7:**e38720. doi:10.1371/journal.pone.0038720.
15. **Ruiz-Barba JL, Cathcart DP, Warner PJ, Jimenez-Diaz R.** 1994. Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in Spanish-style green olive fermentations. Appl. Environ. Microbiol. **60:**2059–2064.
16. **Ruiz-Barba JL, Jimenez-Diaz R.** 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. Food Microbiol. **30:**253–259.
17. **Hurtado A, Reguant C, Bordons A, Rozes N.** 2012. Lactic acid bacteria from fermented table olives. Food Microbiol. **31:**1–8.
18. **Rodriguez H, Curiel JA, Landete JM, de las Rivas B, Lopez de Felipe F, Gomez-Cordoves C, Mancheno JM, Munoz R.** 2009. Food phenolics and lactic acid bacteria. Int. J. Food Microbiol. **132:**79–90.
19. **Landete MJ, Curiel AJ, Rodriguez H, Rivas BDL, Munoz R.** 2008. Study of the inhibitory activity of phenolic compounds found in olive products and their degradation by *Lactobacillus plantarum* strains. Food Chem. **107:**320–326.
20. **Medina E, Brenes M, Romero C, Garcia A, de Castro A.** 2007. Main antimicrobial compounds in table olives. J. Agric. Food Chem. **55:**9817–9823.
21. **Barthelmebs L, Diviès C, Cavin J-F.** 2000. Knockout of the *p*-coumarate decarboxylase gene from *Lactobacillus plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. Appl. Environ. Microbiol. **66:**3368–3375.
22. **Gury J, Seraut H, Tran NP, Barthelmebs L, Weidmann S, Gervais P, Cavin JF.** 2009. Inactivation of PadR, the repressor of the phenolic acid stress response, by molecular interaction with Usp1, a universal stress protein from *Lactobacillus plantarum*, in *Escherichia coli*. Appl. Environ. Microbiol. **75:**5273–5283.
23. **Gury J, Barthelmebs L, Tran NP, Diviès C, Cavin J-F.** 2004. Cloning, deletion, and characterization of PadR, the transcriptional repressor of the phenolic acid decarboxylase-encoding *padA* gene of *Lactobacillus plantarum*. Appl. Environ. Microbiol. **70:**2146–2153.
24. **Licandro-Seraut H, Gury J, Tran NP, Barthelmebs L, Cavin J-F.** 2008. Kinetics and intensity of the expression of genes involved in the stress response tightly induced by phenolic acids in *Lactobacillus plantarum*. J. Mol. Microbiol. Biotechnol. **14:**41–47.

25. **Tran NP, Gury J, Dartois V, Nguyen TK, Seraut H, Barthelmebs L, Gervais P, Cavin JF.** 2008. Phenolic acid-mediated regulation of the *padC* gene, encoding the phenolic acid decarboxylase of *Bacillus subtilis*. J. Bacteriol. **190:**3213–3224.
26. **Duy NV, Mader U, Tran NP, Cavin J-F, Tam le T, Albrecht D, Hecker M, Antelmann H.** 2007. The proteome and transcriptome analysis of *Bacillus subtilis* in response to salicylic acid. Proteomics **7:**698–710.
27. **Mahillon J, Chandler M.** 1998. Insertion sequences. Microbiol. Mol. Biol. Rev. **62:**725–774.
28. **Hayes F.** 2003. Transposon-based strategies for microbial functional genomics and proteomics. Annu. Rev. Genet. **37:**3–29.
29. **Licandro-Seraut H, Brinster S, van de Guchte M, Scornec H, Maguin E, Sansonetti P, Cavin JF, Serror P.** 2012. Development of an efficient *in vivo* system (P_{junc}-TpaseIS*1223*) for random transposon mutagenesis of *Lactobacillus casei*. Appl. Environ. Microbiol. **78:**5417–5423.
30. **Torriani S, Felis GE, Dellaglio F.** 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. Appl. Environ. Microbiol. **67:**3450–3454.
31. **Servili M, Baldioli M, Selvaggini R, Miniati E, Macchioni A, Montedoro G.** 1999. High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pomace and 1D- and 2D-nuclear magnetic resonance characterization. J. Am. Oil Chem. Soc. **76:**873–882.
32. **Servili M, Urbani S, Esposto S, Taticchi A, Veneziani G, Maio I, Sordini B, Selvaggini R, Corsetti A.** 2012. Standardizzazione del processo di deamarizzazione biologica per il miglioramento della qualità delle olive da tavola di varietà italiane, p 109–121. *In* Ed U (ed), Pogettazione, realizzazione e gestione di sistemi di controllo del rispetto delle norme di autenticità, qualità e commercializzazione dell'olio di oliva e delle olive da mensa immessi sul mercato. Tipografia Grafica G&G srl, Marino, Rome, Italy. (In Italian.)
33. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34. **Aukrust TW, Brurberg MB, Nes IF.** 1995. Transformation of *Lactobacillus* by electroporation. Methods Mol. Biol. **47:**201–208.
35. **Dower WJ, Miller JF, Ragsdale CW.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
36. **Heap JT, Pennington OJ, Cartman ST, Minton NP.** 2009. A modular system for *Clostridium* shuttle plasmids. J. Microbiol. Methods **78:**79–85.
37. **Duran MC, Garcia P, Brenes M, Garrido A.** 1993. *Lactobacillus plantarum* survival during the 1st days of ripe olive brining. Syst. Appl. Microbiol. **16:**153–158.
38. **Ruiz-Barba JL, Rios-Sanchez RM, Fedriani-Iriso C, Olias JM, Rios JL, Jimenez-Diaz R.** 1990. Bactericidal effect of phenolic compounds from green olives on *Lactobacillus plantarum*. Syst. Appl. Microbiol. **13:**199–205.
39. **Juven B, Henis Y, Jacoby B.** 1972. Studies on the mechanism of the antimicrobial action of oleuropein. J. Appl. Bacteriol. **35:**559–567.
40. **Newbury SF, Smith NH, Robinson EC, Hiles ID, Higgins CF.** 1987. Stabilization of translationally active mRNA by prokaryotic REP sequences. Cell **48:**297–310.
41. Reference deleted.
42. **Wood JM.** 2011. Bacterial osmoregulation: a paradigm for the study of cellular homeostasis. Annu. Rev. Microbiol. **65:**215–238.
43. **Kramer R.** 2010. Bacterial stimulus perception and signal transduction: response to osmotic stress. Chem. Rec. **10:**217–229.
44. **Tsakalidou E, Papadimitriou K.** 2011. Stress responses of lactic acid bacteria. Springer, New York, NY.
45. **Molenaar D, Bringel F, Schuren FH, de Vos WM, Siezen RJ, Kleerebezem M.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J. Bacteriol. **187:**6119–6127.
46. **Curiel JA, Rodriguez H, de Las Rivas B, Anglade P, Baraige F, Zagorec M, Champomier-Verges M, Munoz R, Lopez de Felipe F.** 2011. Response of a *Lactobacillus plantarum* human isolate to tannic acid challenge assessed by proteomic analyses. Mol. Nutr. Food Res. **55:**1454–1465.
47. **Koponen J, Laakso K, Koskenniemi K, Kankainen M, Savijoki K, Nyman TA, de Vos WM, Tynkkynen S, Kalkkinen N, Varmanen P.** 2012. Effect of acid stress on protein expression and phosphorylation in *Lactobacillus rhamnosus* GG. J. Proteomics **75:**1357–1374.
48. **Di Cagno R, De Angelis M, Limitone A, Fox PF, Gobbetti M.** 2006. Response of *Lactobacillus helveticus* PR4 to heat stress during propagation in cheese whey with a gradient of decreasing temperatures. Appl. Environ. Microbiol. **72:**4503–4514.
49. **Lee K, Lee HG, Pi K, Choi YJ.** 2008. The effect of low pH on protein expression by the probiotic bacterium *Lactobacillus reuteri*. Proteomics **8:**1624–1630.
50. **Castaldo C, Vastano V, Siciliano RA, Candela M, Vici M, Muscariello L, Marasco R, Sacco M.** 2009. Surface displaced alfa-enolase of *Lactobacillus plantarum* is a fibronectin binding protein. Microb. Cell. Fact. **8:**14.
51. **Rozes N, Peres C.** 1998. Effects of phenolic compounds on the growth and the fatty acid composition of *Lactobacillus plantarum*. Appl. Microbiol. Biotechnol. **49:**108–111.
52. **van Bokhorst-van de Veen H, Abee T, Tempelaars M, Bron PA, Kleerebezem M, Marco ML.** 2011. Short- and long-term adaptation to ethanol stress and its cross-protective consequences in *Lactobacillus plantarum*. Appl. Environ. Microbiol. **77:**5247–5256.
53. **Schulze-Gahmen U, Pelaschier J, Yokota H, Kim R, Kim SH.** 2003. Crystal structure of a hypothetical protein, TM841 of *Thermotoga maritima*, reveals its function as a fatty acid-binding protein. Proteins **50:**526–530.
54. **Gibson TJ.** 1984. Studies on the Epstein-Barr virus genome. Ph.D. thesis. Cambridge University, Cambridge, England.
55. **Rochat T, Bouloc P, Repoila F.** Gene expression control by selective RNA processing and stabilization in bacteria. FEMS Microbiol. Lett., in press. doi:10.1111/1574-6968.12162.
56. **Maldonado-Barragan A, Caballero-Guerrero B, Lucena-Padros H, Ruiz-Barba JL.** 2011. Genome sequence of *Lactobacillus pentosus* IG1, a strain isolated from Spanish-style green olive fermentations. J. Bacteriol. 193:5605.